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ORIGINAL ARTICLE

Plasma granzyme B as a predicting factor of coronary artery disease—Clinical significance in patients with chronic renal failure

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Summary
Objectives: To elucidate the role of granzyme B in coronary artery disease (CAD) in patients with chronic kidney disease (CKD). We hypothesized that granzyme B plays an important role in the formation of coronary artery lesions in patients with CKD.
Patients and methods: We studied 141 patients (116 men and 25 women; mean age, 64.2 ± 9.6 years) and 16 control subjects. Diagnosis of CAD was confirmed by selective coronary angiography. CKD was defined as a sustained decrease in the estimated glomerular filtration (eGFR) rate less than 60 mL/min/1.73 m^2 over 3 months. We assigned patients to three groups: CAD without CKD (CAD group, $n = 46$), CKD without CAD (CKD group, $n = 18$), and CAD with CKD (CAD/CKD group, $n = 77$). Plasma granzyme B was measured by enzyme-linked immunosorbent assay. Factors contributing to the severity of CAD were analyzed by multiple regression analysis in patients with CAD.
Results: Plasma levels of high-sensitivity CRP (hs-CRP) and granzyme B in the CAD/CKD group were significantly higher than in other groups. A significant positive correlation was observed between plasma hs-CRP and granzyme B levels. A significant negative correlation was observed between eGFR and granzyme B levels. Multiple regression analysis revealed that granzyme B and hs-CRP levels were independent predicting variables of the number of stenoses in major coronary arteries.

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Conclusions: These results indicate that granzyme B might be a novel risk factor for the formation of coronary atherosclerosis by inducing apoptosis of vascular tissues in patients with CKD.

Introduction

A number of epidemiological studies have reported that chronic kidney disease (CKD) is an independent risk factor for cardiovascular diseases [1—3]. Cardiovascular mortality is highest in patients with end-stage renal disease; however, a significant increase in cardiovascular events has been observed even among patients with mild renal insufficiency [1]. It has also been reported that clinical outcome of percutaneous coronary intervention is not sufficient enough in patients with end-stage renal disease [2]. Although a number of reports have demonstrated increased cardiovascular mortality in CKD, the precise mechanisms of how CKD takes part in the onset of cardiovascular diseases are still not fully understood. It has been speculated that hypertension, dyslipidemia, malnutrition, homocysteine, oxidative stress, and inflammation are involved in the progression of atherosclerosis in patients with CKD [3—7]. Among humoral factors, interleukins, tumor necrosis factors, and transforming growth factors are possible candidates to promote atherosclerotic processes in patients with CKD by triggering endothelial dysfunction [8—11].

Recently, we have reported that the production of granzyme B from peripheral blood mononuclear cells was increased in patients with acute coronary syndrome [12]. Granzyme B is a member of the serine protease family released from cytotoxic lymphocytes and plays an important role in cellular apoptosis by activating intracellular caspases. Granzyme B induces cell death by mechanisms such as the activation of caspases, degradation of structural proteins, and directing the proapoptotic molecule Bid to the mitochondrial compartment [13,14]. Chamberlain et al. have reported that granzyme B might play important roles in the progression of atherosclerosis in patients receiving heart transplant surgery via the induction of endothelial apoptosis [15,16]. It is possible that proapoptotic molecules are involved in the formation of atherosclerosis in a group of patients with immunological disorders [17]. Interestingly, patients with chronic renal failure are reported to have various immunological disorders that are significant contributing factors for long-term survival [18—20].

High-sensitivity C-reactive protein (hs-CRP) is useful to evaluate high-risk CKD patients with coronary atherosclerosis, however non-specific elevation might reduce the sensitivity and specificity of this marker. We hypothesized that granzyme B might also be useful to detect coronary atherosclerosis in patients with CKD in addition to hs-CRP. We have measured the plasma levels of granzyme B in patients with coronary artery disease (CAD) with or without CKD to examine the role of granzyme B in CAD patients accompanied by CKD.

Subjects and methods

The Ethics Committee of Jichi Medical University approved the protocol of this study. All patients enrolled in this study gave informed consent.

Patients

Consecutive patients who underwent coronary angiography in Jichi Medical University Hospital between October 2006 and October 2007 were included in this study. We studied 141 patients (116 men and 25 women, aged 64.2 ± 9.6 years, ranging from 38 to 81 years) who underwent coronary angiography in our hospital. ''CAD'' patients were defined as patients showing significant coronary stenosis diagnosed by selective coronary angiography. ''CKD'' patients were defined as patients who showed sustained proteinuria (over 2+ by semi-quantification method) for more than three months and/or an estimated glomerular filtration rate (eGFR) less than 60 mL/min/1.73 m^2 according to the US National Kidney Foundation [21]. Briefly, eGFR was calculated using the simplified prediction equation described below derived from the collaborators developing the Japanese equation for eGFR [22]:

$$\mathrm{eGFR\,(mL/min/1.73\,m^2)} = 194 \times \mathrm{creatinine}^{-1.094} \times \mathrm{age}^{-0.287} (\times 0.739 \text{ if female}).$$

We divided patients into three groups according to the presence of CAD and CKD; CAD group (CAD patients without CKD, $n = 46$), CKD group (CKD patients without CAD, $n = 18$), and CAD/CKD group (CAD patients with CKD, $n = 77$). The CKD group included 2 patients with valvular heart disease, 1 aortic aneurysm, 1 thoracic aneurysm, and 14 chest pain patients without the evidence of coronary atherosclerosis or variant angina. Patients without CAD or CKD ($n = 16$, 10 men and 6 women, aged 65.0 ± 8.2 years, ranging from 38 to 81 years) were also recruited to the study as a control group. All control subjects underwent coronary angiography because of chest pain. No significant coronary stenosis was observed in control subjects.

We excluded patients with acute coronary syndrome, acute inflammatory disease, acute renal failure, hematological disorder, malignancy, and patients taking immunosuppressive medicine. Hypertension, diabetes mellitus, and dyslipidemia were diagnosed according to the criteria described by Ninomiya et al. [23]. Hs-CRP was measured by the latex nephelometry method described by Ledue et al. [24].

Measurement of plasma granzyme B levels

The concentration of granzyme B in plasma was determined using enzyme-linked immunosorbent assay kits according to the manufacturer's instructions (Kamiya Biomedical Company, Seattle, WA, USA). The lower limit of granzyme B detection was 1.3 pg/mL. Intra-assay variation of the kit is 6.5% and inter-assay variation less than 10%.

Statistical analysis

All values are expressed as the means ± SEM unless otherwise indicated. The significant difference in proportion was examined by the chi-square test. The significance of differences among the three groups was determined by one-way analysis of variance. If data did not follow Gaussian distribution, the significance of the three groups was determined by the Kruskal—Wallis test. The relationship between two variables was examined by calculating Pearson's correlation coefficient and the corresponding p values. Multiple regression analysis was conducted with computer software (SPSS version 16.0, Chicago, IL, USA). Values of $p < 0.05$ were considered significant.

Results

Baseline characteristics of subjects

Table 1 shows the baseline characteristics of the study subjects. As shown in Table 1, mean age, gender, and the incidence of coronary risk factors were not significantly different among the three groups. Left ventricular ejection fraction in the CKD group and CAD/CKD groups was significantly lower than in the CAD group. The hs-CRP in

Table 1 Baseline characteristics of the three study groups.

	CAD group	CKD group	CAD/CKD group
n	46	18	77
Age (years old)	62.7 ± 9.9	61.3 ± 12.4	65.7 ± 8.4
Gender (male/female)	41/5	13/5	62/151
Hypertension	36 (78.3%)	12 (66.7%)	68 (88.3%)
Dyslipidemia	29 (63.0%)	6 (33.3%)	41 (53.2%)
Diabetes mellitus	20 (43.5%)	7 (38.9%)	41 (53.2%)
Smoking	25 (54.3%)	9 (50.0%)	41 (53.2%)
Family history of CAD	13 (28.3%)	1 (0.06%)	18 (23.3%)
Vessel disease[a]	1/23/10/12	18/0/0/0	3/37/15/22
LVEF	$65.7 \pm 1.4\%$	$51.6 \pm 3.7\%$[∫]	$58.5 \pm 1.6\%$[¶]
History of OMI	6 (13.0%)	0 (0%)	17 (22.0%)
eGFR (mL/min/1.73 m^2)	76.7 ± 13.2	14.7 ± 12.5[‡]	27.1 ± 21.1[‡]
hs-CRP (ng/mL)	2420 ± 763	2818 ± 1156	7294 ± 2193[†]

CAD, coronary artery disease; CKD, chronic kidney disease; LVEF, left ventricular ejection fraction; OMI, old myocardial infarction; eGFR, estimated glomerular filtration rate; hs-CRP, high-sensitivity C-reactive protein.

[∫] $p < 0.001$ versus CAD group.

[¶] $p < 0.01$ versus CAD group.

[‡] $p < 0.001$ compared with CAD group.

[†] $p < 0.01$ compared with CAD group (Kruskal—Wallis test).

[a] 0-/1-/2-/3-vessel disease and lesions including left main trunk.

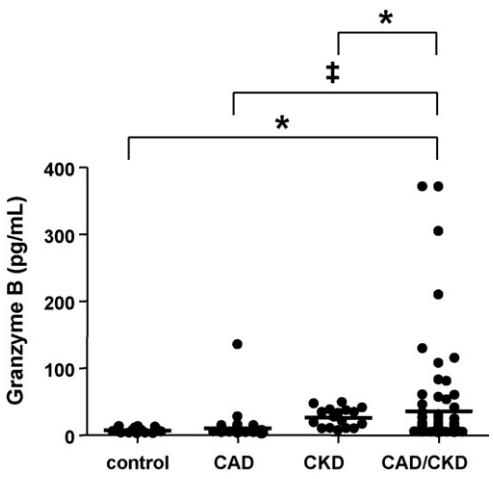

Figure 1 Levels of plasma granzyme B in study patients. The Kruskal–Wallis test revealed that plasma levels of granzyme B in CAD/CKD patients were significantly higher than those in control subjects, patients with CAD or patients with CKD (control, 7.1 ± 1.0 pg/mL; CAD, 10.6 ± 2.8 pg/mL; CKD, 27.3 ± 3.3 pg/mL; CAD/CKD, 35.5 ± 8.1 pg/mL; $p < 0.001$). *$p < 0.05$, ‡$p < 0.001$. CAD, coronary artery disease; CKD, chronic kidney disease.

the CAD/CKD group was significantly higher than that in CKD or CAD group. As shown in Figure 1, plasma granzyme B levels in the CAD/CKD group were significantly higher than in the control group, CAD, or CKD group (control, 7.1 ± 1.0 pg/mL; CAD, 10.6 ± 2.8 pg/mL; CKD, 27.3 ± 3.3 pg/mL; CAD/CKD, 35.5 ± 8.1 pg/mL; $p < 0.001$).

Correlation between granzyme B and other parameters

As shown in Figure 2A, there was a significant negative correlation between plasma granzyme B levels and eGFR ($r = -0.33$, $p < 0.001$). Similarly, a significant positive correlation was observed between plasma granzyme B levels and creatinine levels ($r = +0.27$, $p = 0.001$). There was a positive correlation between plasma granzyme B levels and hs-CRP with marginal significance ($r = +0.39$, $p = 0.07$). As shown in Figure 2B, a significant positive correlation was observed plasma granzyme B levels and logarithmic (log)-transformed hs-CRP ($r = +0.23$, $p < 0.01$). Next, we conducted multivariate regression analysis to examine the factors contributing to plasma granzyme B levels. We set plasma granzyme B levels as a responsible variable and age, sex, body mass index, hypertension, dyslipidemia, diabetes mellitus, current smoker, family history of CAD, eGFR, and log-transformed hs-CRP as explanatory variables. As shown in Table 2, diabetes mellitus and eGFR were significant factors contributing to plasma granzyme B levels ($F = 0.310$, $p = 0.002$).

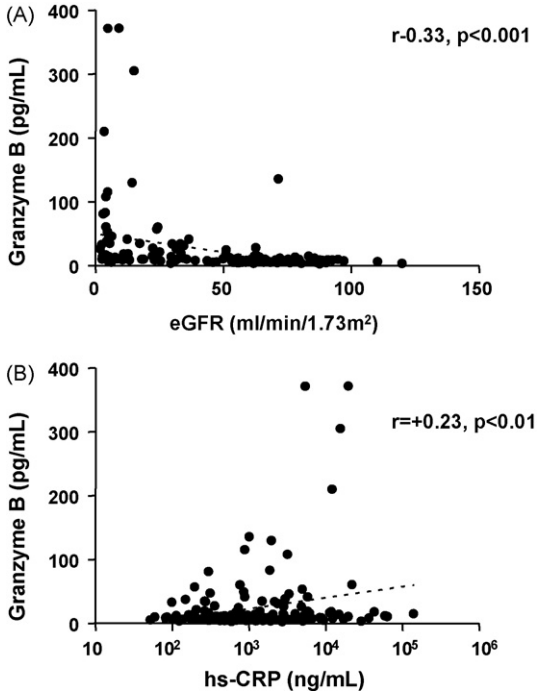

Figure 2 Simple correlations between plasma granzyme B levels and clinical parameters. Panel A: correlation between plasma granzyme B and estimated glomelular filtration rate (eGFR). A significant negative correlation was found between these parameters ($r = -0.33$, $p < 0.001$). Panel B: a significant positive correlation was found between plasma granzyme B levels and log-transformed high-sensitivity C-reactive protein (hs-CRP) levels ($r = +0.23$, $p < 0.01$).

Multivariate regression analysis for severity of coronary artery disease

We examined the independent contributing factors for the severity of CAD in patients with CAD

Table 2 Predicting factors for plasma granzyme B levels.

	β coefficient	p value
Age	+0.031	0.693
Sex	−0.130	0.113
Body mass index	−0.038	0.640
Hypertension	−0.012	0.884
Dyslipidemia	−0.143	0.069
Diabetes mellitus	+0.202	0.015
Current smoker	−0.042	0.614
Family history	+0.080	0.290
eGFR	−0.275	0.001
hs-CRP	+0.108	0.176

eGFR, estimated glomerular filtration rate; hs-CRP, high-sensitivity C-reactive protein.

(CAD and CAD/CKD groups). The number of significant stenoses in the major coronary artery was used as the responsible variable (zero-vessel disease, 0; one-vessel disease, 1; two-vessel disease, 2; three-vessel disease and lesions including left main trunk, 3). We established three models to clarify the predicting factor for the severity of CAD by setting age, sex, body mass index, hypertension, dyslipidemia, diabetes mellitus, current smoker, family history of CAD, eGFR, log-transformed hs-CRP level, and plasma granzyme B levels as explanatory variables. Model 1 includes patients' demographic data and plasma granzyme B levels. Model 2 includes classic risk factors for atherosclerosis as explanatory variables in addition to the Model 1. Model 3 includes body mass index, eGFR, and log-transformed hs-CRP as explanatory variables in addition to the Model 2. As shown in Table 3, we found that the plasma granzyme B level and hs-CRP were significant predicting factors for severity of CAD in all models.

Table 3 Predicting factors for severity of coronary artery disease.

	β coefficient	*p* value
Model 1: $F = 346$, $p = 0.019$		
Age	+0.093	0.300
Sex	+0.041	0.645
Plasma granzyme B levels	+0.258	0.004
Model 2: $F = 2.02$, $p = 0.050$		
Age	+0.108	0.240
Sex	+0.040	0.682
Hypertension	+0.022	0.810
Dyslipidemia	−0.031	0.741
Diabetes mellitus	+0.159	0.113
Current smoking	+0.110	0.258
Family history of CAD	−0.201	0.811
Plasma granzyme B levels	+0.207	0.029
Model 3: $F = 2.06$, $p = 0.029$		
Age	+0.094	0.305
Sex	+0.035	0.725
Body mass index	−0.070	0.468
Hypertension	+0.034	0.718
Dyslipidemia	−0.006	0.948
Diabetes mellitus	+0.135	0.179
Current smoking	+0.117	0.228
Family history of CAD	−0.017	0.851
eGFR	+0.121	0.223
hs-CRP (log-transformed)	+0.197	0.044
Plasma granzyme B levels	+0.195	0.049

eGFR, estimated glomerular filtration rate; hs-CRP, high-sensitivity C-reactive protein; CAD, coronary artery disease.

Comparison of plasma granzyme B levels between patients with or without hemodialysis

Because a significant negative correlation was observed between plasma granzyme B levels and eGFR, we examined granzyme B levels in patients with end-stage renal disease. We compared plasma granzyme B levels in patients with or without hemodialysis (HD). We divided CAD/CKD patients according to HD treatment; CAD/CKD with HD group ($n = 33$) and CAD/CKD without HD group ($n = 44$). There were no significant differences in terms of age, sex, and coronary risk factors between two groups. Plasma granzyme B levels in the CAD/CKD with HD group were significantly higher than in the CAD/CKD without HD group (67.4 ± 17.7 pg/mL versus 12.2 ± 1.9 pg/mL, $p < 0.001$). We did not find a significant difference in granzyme B levels in the CAD/CKD group without HD and CKD group without HD (12.2 ± 1.9 pg/mL versus 21.9 ± 3.8 pg/mL).

Discussion

The present study has shown that the plasma granzyme B level was elevated in CAD patients with CKD. Univariate analysis showed that plasma granzyme B is related to renal function and inflammation. Multivariate regression analysis disclosed that diabetes mellitus and eGFR were significant factors for the elevation of plasma granzyme B levels. Interestingly, multivariate regression analysis has revealed that the plasma granzyme B level is a significant independent factor for the severity of CAD together with hs-CRP. In addition, plasma granzyme B was markedly elevated in CAD patients receiving HD.

Elevation of circulating inflammatory cytokines, decreased anti-inflammatory cytokines, and activation of lymphocytes have been reported in patients with chronic kidney disease [25—27]. We focused on the perforin/granzyme system in CAD patients with CKD because a previous report has shown the activation of T lymphocytes in patients with renal failure [25]. Normally, the perforin/granzyme system induces apoptosis of infected cells and cancer cells. It is reported that granzyme B injures endothelial cells in patients with immunological dysregulation, such as recipients of allograft transplantation [28]. It has also been reported that administration of a serine protease inhibitor attenuated atheromatous plaque formation in apoE-knockout mice [29] and vascular injury after allograft transplantation [30]. Thus, it

is possible that serine proteases play an important role in atheromatous plaque formation in certain group of patients.

We have observed that phorbol-12-myristate-13-acetate, a protein kinase C activator, upregulated the release of granzyme B from cultured peripheral blood mononuclear cells; thus, a protein kinase C-mediated mechanism is at least partially involved in granzyme B expression [12]. It has been reported that patients with diabetes mellitus are under the condition of increased cellular protein kinase C activity. Conversely, granzyme B might play an important role in patients with diabetic angiopathy.

We found that plasma granzyme B and hs-CRP were independent factors for the severity of CAD. Our data also showed that decreased renal function is a factor in the elevation of plasma granzyme B levels. Interestingly, eGFR was not a predicting factor for the severity of CAD; thus, it is possible that granzyme B is an important mediator to connect renal dysfunction and coronary atherosclerosis in patients with CKD.

CAD/CKD patients receiving hemodialysis showed extremely high plasma granzyme B levels and it is possible that cytotoxic lymphocytes are activated in patients receiving hemodialysis. This immunological disorder might be evoked by direct contact of circulating blood cells with the hemodialysis membrane. Proinflammatory molecules that cannot be eliminated by hemodialysis might also upregulate circulating granzyme B levels in patients receiving HD.

In conclusion, we have demonstrated that granzyme B is a novel risk factor for coronary atherosclerosis in CKD patients, particularly in end-stage renal disease. The plasma granzyme B level could be a marker to detect high-risk patients with cardiovascular complications in CKD patients in addition to hs-CRP. Inhibition of granzyme B could prevent coronary atherosclerosis in certain groups of patients.

Study limitations

First, we calculated the eGFR value in patients receiving HD. The serum creatinine level changes drastically by HD. Thus eGFR in patients receiving HD might not be accurate. We have to take into account this point in interpretation of data. We could not measure plasma levels of intrinsic granzyme B inhibitor, protease inhibitor 9 in our study patients; therefore, we did not know plasma granzyme B activity in our study patients. We have tried to measure plasma granzyme B activity using a fluorescent or colorimetric substrate; however, we could not obtain reproducible results in the present study. In addition, a prospective study with a large population of patients should be performed to elucidate the clinical significance of granzyme B in future cardiovascular events.

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